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RESEARCH ARTICLE

Synthesis and Characterization of Low Molecular Weight Linear Polyethylenimines for Gene Delivery

Majed Matar*, Gregory Slobodkin, Angela Rea-Ramsey, Elaine Brunhoeber, Jeanette L. Skoyen, Jason G. Fewell, Danny H. Lewis, and Khursheed Anwer

Expression Genetics, Inc., Huntsville, AL 35801, USA

The therapeutic application of commercially available polyethylenimines (e.g., PEI 25 kD) is marred by substantial toxicity. The benefit of using lower molecular weight polyethylenimines (<25 kD) has not been fully explored because of limited availability and high molecular heterogeneity (e.g., degree of branching) among the available few. We have synthesized a series of low molecular weight linear polyethylenimines (LPEI; 2.5–25 kD) with a single synthesis scheme to minimize molecular heterogeneity. DNA formulation with the newly synthesized linear polyethylenimines resulted in the formation of stable nanoparticles (100–150 nm) of positive zeta potential. Addition of these nanoparticles onto COS-1 and HEK 293 cell cultures led to transgene expression the efficiency and cytotoxicity of which varied with the LPEI size. The lowest molecular weight LPEI (LPEI 2.5 kD) gave the smallest level of gene expression and did not exert any cytotoxicity. The transfection activity exponentially increased with higher molecular weight LPEIs reaching maximal level with 7.5 kD LPEI and was accompanied with some cytotoxicity. The transfection activity of 7.5 kD LPEI was equal to that of the higher molecular weight LPEIs including 25 kD LPEI, but caused less cytotoxicity. To achieve high transfection efficiency without substantial increase in cytotoxicity, we cross-linked LPEI 3.6 kD with a biodegradable linkage to form a multi-block copolymer (BD3.6K) of approximately 8 kD. The multi-block copolymer, BD3.6K, expressed 20-fold higher transfection activity than that of the monomer block and produced significantly lower cytotoxicity than 25 kD PEI *in vitro*. Following intravenous administration, plasmid/BD3.6K complexes elicited significant gene transfer in lungs, while complexes prepared with monomer block did not yield discernable transfection activity. The transfection efficiency of the systemically administered plasmid/BD3.6K complexes was 2.5-times and 70-times higher than that of linear and branched 25 kD PEI, respectively. Transfection complexes prepared with BD3.6K exhibited better tolerability than complexes prepared with 25 kD PEIs. These results demonstrate that: (1) the lower molecular weight linear polyethylenimines (<10 kD) are more suitable for gene delivery than the commercially available higher molecular weight polyethylenimines (25 kD) and (2) the cross-linking of the non-toxic low molecular weight polyethylenimines via biodegradable linkage is a viable approach to improving PEI transfection efficiency without significantly increasing the cytotoxicity.

Keywords: BD3.6K, Polyethylenimines, PEI, Biodegradable Polymer, Cross-Linking, Systemic Gene Delivery, *In Vitro* Transfection, Cytotoxicity.

1. INTRODUCTION

The success of gene therapy relies on the ability of the delivery systems to promote gene transfer to the target tissues with high efficiency and minimal toxicity. Non-viral gene delivery systems are particularly suitable because of their simple design and lack of a specific immune response.[1] The interest in polymeric gene carriers is growing because of their flexible chemical design that allows for incorporation of various functional moieties required to overcome the delivery barriers.[2–6] Polyethylenimine (PEI) is a widely used gene delivery polymer with inherent endosomolytic activity that gives this polymer a distinct advantage over conventional non-viral gene delivery systems.[7,8] Despite its wide use in non-clinical studies, the clinical development of the commonly used polyethylenimine, PEI 25 kD, has been sluggish due to acute toxicity.[9–11] For example, intravenous administration of PEI-DNA complexes into mice caused acute systemic

*Author to whom correspondence should be addressed.

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toxicity including activation of lung endothelium, adhesion of the complexes to CD 11-b positive cells and platelet aggregation.[9] Intrauterine delivery of PEI-DNA complexes into mouse fetal liver caused systemic toxicity and higher fetal mortality.[11] When different size fractions of a heterogeneous commercial preparation of 25 kD PEI were compared, the fraction containing the lower molecular weight polyethylenimines expressed higher transfection activity and lower cytotoxicity than that of the starting material.[12] Fischer et al. reported a 12 kD branch PEI, which gave higher transfection activity, and lower cytotoxicity as compared to a much heavier (1616 kD) branch PEI.[13] These studies underscore the importance of low molecular weight polyethylenimines (<25 kD) and warrant a systematic evaluation and characterization thereof. Unfortunately, few commercial polyethylenimines of molecular weight below 25 kD are available and are notoriously heterogeneous in molecular configuration from strictly linear to highly branched. Therefore, in order to achieve an accurate assessment of the full potential of PEI based delivery systems, it is imperative to synthesize a wide range of low molecular weight polyethylenimines with a single synthesis scheme to minimize molecular heterogeneity and then analyze them for transfection activity and cytotoxicity.

Other groups have sought to improve gene delivery efficiency while minimizing cytoxicity by using low molecular weight PEI (0.8 kD) cross linked with dithiobis (succinimidylpropionate) (DSP) or dimethyl · 3,3′-dithiobispropionimidate · 2HCl (DTBP).[14] When tested in CHO cells it was shown that gene transfer using the cross-linked polymers was enhanced compared to 0.8 kD PEI, but there was no improvement over high molecular weight 25 kD. Additionally, the elevated expression levels were correlated with higher toxicity in three of four crossed linked polymers tested.

Recently Thomas et al.[15] have reported a similar approach to enhance transfection efficiency and lower toxicity of polyethylenimines. An improvement in transgene expression was observed when using cross-linked small PEIs (mixture of 2 kD and 0.423 kD) as compared to 25 kD PEI in COS-7 cell cultures and in mouse lungs after intratracheal administration. However, the toxicity resulting from intratracheal administration was not determined.

In the present study, we have synthesized a series of low molecular weight linear polyethylenimines (LPEI) using a uniform synthesis scheme to minimize molecular and structural heterogeneity, and compared them for transfection activity and cytotoxicity. Linear polyethylenimines were used as compared to the branched polyethylenimines in order to minimize cytotoxicity.[16] A non-toxic low molecular weight LPEI was selected from the above series and cross-linked with a biodegradable linker, producing a multi-block copolymer of higher transfection activity and lower cytotoxicity relative to commercially available 25 kD PEI.

2. MATERIALS AND METHODS

2.1. Materials

2-phenyloxazoline and thionyl chloride was purchased from Aldrich; dimethylsulfate, sulfuric acid, potassium hydroxide, sodium hydroxide, sodium sulfate, barium chloride, 3,3′-dithiodipropionic acid, trifluoroacetic acid, *t*-butyldicarbonate, hydrochloric acid, methylene chloride, chloroform, acetone, hexane, and toluene from Acros; 25 kD branched PEI from Sigma-Aldrich and Linear 25 kD from Polysciences; Dulbecco's modified eagle media, fetal bovine serum, and trypsin from MediaTech; 12-well plates from BD Falcon, COS-1 and HEK 293 cells from ATCC and ExGen 500 from Fermentas. All other chemicals were reagent grade.

2.2. Synthesis of Linear Polyethylenimines

Linear polyethylenimines were prepared using a modification of Tanaka's method.[17] Briefly, 2-phenyl-2-oxazoline was polymerized by heating at 140 °C for 48 hours in the presence of the initiator, Me_2SO_4. The initiator concentration was varied to obtain different size polyethylenimines. The molecular mass of the polymerized benzoylethyleneimine was determined by gel permeation chromatography using polystyrene standards. The polybenzoylethyleneimine was debenzoylated by heating with 60% H_2SO_4 at 140–150 °C for 16–20 hours. After removal of the benzoic acid byproduct by steam distillation, the corresponding LPEI sulfate salt was separated from the reaction mixture by cooling. The free base or chloride forms of LPEI were obtained by treatment of LPEI sulfate with NaOH or $BaCl_2$, respectively. The integrity of the polyethylenimine backbone upon acid hydrolysis was examined by rebenzoylation of the product and comparing its molecular mass to that of the corresponding pre-hydrolysis benzoylated intermediate. Reduction in molecular weight would indicate the polymer degradation during acid hydrolysis.

2.3. Synthesis of Cross-Linked Multi-Block Polyethylenimine

A multi-block copolymer of 3.6 kD LPEI was synthesized by cross-linking with dithiodipropionyl linker and named BD3.6K. Dithiodipropionyl dichloride was prepared by mixing 33.3 mM dithiodipropionic acid with 106 mM thionyl chloride and stirring the mixture at room temperature for 5 days followed by removal of the solids by filtration and evaporation of the residual thionyl chloride under vacuum. 48.8 mM of dehydrated 3.6 kD LPEI was reacted overnight with 46.4 mM of t-Butoxycarbonyl (BOC) anhydride. The reaction product, $LPEI_{3600}BOC$, was vacuum concentrated, triturated with hexane, dissolved in 25 ml of chloroform and then 2.1 mM dithiodipropionyl chloride

was added to it under constant stirring. The mixture gelled after 36 hours, indicating polymer cross-linking. At this point 20 ml of trifluoroacetic acid was added to remove the BOC groups and the mixture was stirred for 30 min. The lower layer of the resulting heterogeneous mixture was separated and diluted with 40 ml of water. Residual chloroform and a small amount of particulate impurities were removed by centrifugation. An aqueous solution of Na_2SO_4 (10%) was added to the supernatant, and the resulting off-white precipitate of the cross-linked BD3.6K sulfate was collected, washed with water and then with acetone before drying. The poorly soluble sulfate salt was converted into readily soluble chloride form by treatment with barium chloride in aqueous solution, insoluble barium sulfate was removed by filtration.

2.4. Estimation of the Molecular Weight of Cross-Linked Multi-Block Polyethylenimine

The molecular weight of the cross-linked multi-block copolymer BD3.6K was determined by measuring the solution viscosity. Aqueous solutions of various linear polyethylenimines and BD3.6K were prepared at 5 mg/ml in distilled water and placed in a Cannon-Fenske routine viscometer. The flow time of a fixed volume of LPEI solutions and that of the solvent (distilled water) was measured through the viscometer capillary tube. The dimensionless ratio of solution flow time to solvent flow time was recorded as relative viscosity.

2.5. Preparation of Plasmid

The DNA plasmids, pCMV-luc, and pDNR-CMV-lacZ, encoding luciferase and β-galactosidase genes, respectively, were amplified in JM109 *E. coli* strain and purified using Qiagen (Chatsworth, CA) EndoFree Plasmid Maxi-prep or Giga-prep kits according to the manufacturer's instructions. Following purification, the DNA concentration was determined using a UV spectrophotometer at 260 nm. Plasmid DNA integrity was evaluated using agarose gel electrophoresis followed by ethidium bromide staining.

2.6. Preparation and Characterization of Water Soluble LPEI-DNA Complexes

Water soluble complexes of plasmid DNA and linear polyethylenimines (2.5, 3.6, 7.5, 11, 15, and 25 kD) or multi-block cross-linked polymer, BD3.6K, were prepared by mixing a specified amount of luciferase or β-galactosidase plasmid with the polymer at various nitrogen to phosphate (N : P) ratios in 10% lactose solution, and the mixture was incubated at room temperature for 15 minutes to allow the formation of nanocomplexes. The electrophoretic mobility of the LPEI-DNA complexes was determined by agarose gel electrophoresis at 100 V for one hour. The particle size and zeta potential of the nanocomplexes in Milli-Q water was determined at 657 nm at a constant angle of 90° by dynamic light scattering using a 90Plus/BI-MAS Particle sizer with BI-Zeta option (Brookhaven Instruments Corp., Holtsville, NY). To determine if polymer complexation protects the DNA from nuclease degradation, the polymer-DNA complexes were incubated with 5 IU of DNase I in digestion buffer (40 mM Tris-HCl, 10 mM NaCl, 5 mM $MgSO_4$) for 60 minutes at 37 °C. The reaction was terminated with an equal volume of termination buffer (50 mM EDTA, 20 mM Tris-HCl, 1% SDS). Following reaction termination, the samples were incubated with 50 μg of dextran sulfate for 10 minutes at room temperature to release DNA from the polymer. The DNA integrity was examined using 1% agarose gel electrophoresis at 100 V for one hour.

2.7. *In Vitro* Gene Transfer

The ability of the linear polyethylenimines and BD3.6K to promote gene transfer was examined in COS-1 and HEK 293 cells. Cell cultures were prepared to 80% confluency in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS. Transfection complexes containing 1 μg of plasmid DNA were added into each well in serum-free medium for 6 hours. The transfection medium was removed and cells were incubated for an additional 40 hours in serum containing medium. Cell lysate was prepared and analyzed for luciferase activity using a micro plate luminometer (Berthold Detection Systems USA, Oak Ridge, TN). The efficiency of gene transfer was determined with a β-galactosidase plasmid. Cells were transfected with LPEI-β-gal plasmid complexes as described above and β-galactosidase positive cells were visualized with the X-Gal assay (Gene Therapy Systems, Inc., San Diego, CA).

2.8. *In Vivo* Gene Transfer

The transfection complexes containing luciferase plasmid and multi-block copolymer, BD3.6K, were administered into the tail vein of ICR mice (Harlan, Houston, TX). The plasmid concentration in the formulation was fixed at 0.1 mg/ml to yield a 30 μg dose in 0.3 ml injection volume. Lungs were harvested 24 hours after gene injection, homogenized in 1 ml TENT buffer (10 mM Tris, 1 mM EDTA, 100 mM NaCl, 0.5% Triton X-100) and assayed for luciferase activity.

2.9. Measurement of Toxicity

The cytotoxicity of gene transfection *in vitro* was determined by a cell proliferation assay (Promega Corporation, Madison, WI). 20 μL of CellTiter 96® reagent was added to each well after cell transfection, the optical density of the colored product was read after 4 hours at 490 nm to

calculate the cell viability. In some experiments BCA™ protein assay was used to determine cell viability. 20 μl of the transfected cell lysate was analyzed for total protein according to manufacturer's instructions (Pierce Chemical, Rockford, IL). For *in vivo* tolerability, the transfection complexes were administered intravenously into normal ICR mice by tail vein injection. Twenty-four hours after treatment, the animals were sacrificed and examined for gross pathology. Blood samples were collected by retro-orbital bleed and analyzed for clinical chemistry at IDEX Corporation.

3. RESULTS AND DISCUSSION

3.1. Synthesis of Linear Polyethylenimines

Linear polyethylenimines of different molecular weight were synthesized by phenyl-oxazoline polymerization. The chemical composition was verified by NMR spectroscopy. Benzoylated polyethylenimine intermediates ranging from 8,000 to 51,000 Dalton were synthesized by controlling the initiator concentration in the reaction mixture. Polyethylenimine sulfates were obtained by debenzoylation of the intermediates by acid hydrolysis. The molecular weight of the final polyethylenimine salt was obtained by subtraction of the benzoyl weight from the corresponding benzoylated intermediate. The estimated molecular weights of the final LPEI products were 2.5 kD, 3.6 kD, 7.5 kD, 11 kD, 15 kD, and 25 kD. The molecular weights of benzoylated polymer before and after acid hydrolysis were in good agreement, suggesting that the acid hydrolysis was not deleterious to the polymer backbone. The intrinsic viscosity of the aqueous solutions of polyethylenimine salts was determined and plotted against the corresponding molecular weight (Fig. 1). A linear relationship shows that the size distribution of the LPEI series is in good agreement with its anticipated physicochemical properties. This linear relationship was later used to calculate the molecular weight of multi-block copolymer from its viscosity.

3.2. Physico-Chemical Properties and Transfection Activity of Linear Polyethylenimines

The ability of polyethylenimines to interact with plasmid DNA and to promote gene transfer was examined in COS-1 cells. The LPEI-DNA complexes were formed by mixing the polymer and DNA solutions under controlled conditions. The mean particle size and zeta potential of the LPEI-DNA complexes was dependent on the polymer to DNA ratio (Table I). The particle size decreased as the N : P ratio increased indicating stronger compaction of LPEI-DNA complexes at higher polymer concentration. At a fixed N : P ratio the mean particle size of LPEI-DNA complexes was not affected by the polymer size since the total nitrogen content of the polymer did not change

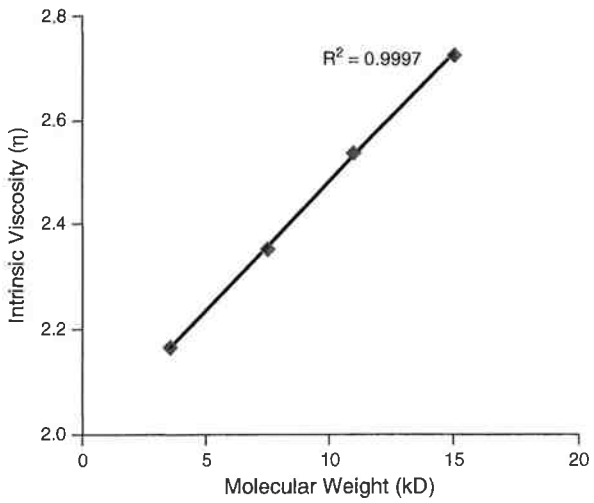

Fig. 1. Linear relationship between molecular weight and intrinsic viscosity of synthetic linear polyethylenimines. Aqueous solutions of various linear polyethylenimines and BD3.6K were prepared at 5 mg/ml in distilled water and placed in a Cannon-Fenske routine viscometer. The flow time of a fixed volume of LPEI solutions and that of the solvent (distilled water) through the viscometer capillary tube was measured. The dimensionless ratio of solution flow time to solvent flow time was recorded as the relative viscosity. The relationship between intrinsic viscosity (ratio of relative viscosity to the concentration of solution) and molecular weight was plotted.

under these conditions. Kunath et al. have reported the formation of larger particles with lower molecular weight polyethylenimines.[18] This difference between our results and those of Kunath et al. might be due to the buffer composition used for particle size analysis. Kunath et al. used NaCl while we have used 10% lactose. It is possible that DNA complexes containing low molecular weight polyethylenimines might be less stable and more easily aggregate in salt solutions.

The gene transfer studies show pronounced enhancement of transfection activity and cytotoxicity with higher molecular weight LPEIs (Fig. 2). The lowest molecular weight LPEI (LPEI 2.5 kD) was non-toxic but showed very low transfection efficiency. Transfection activity was exponentially enhanced as the molecular mass increased

Table I. Comparison of particle size and zeta potential of transfection complexes prepared with various linear polyethylenimines.

LPEI (kD)	N : P ratio	Particle size (nm)	Zeta Potential ζ(mv)	LPEI (kD)	N : P ratio	Particle size (nm)	Zeta Potential ζ(mv)
2.5	3:1	190.4	15.6	11	3:1	140.4	13.7
	10:1	178.8	24.3		10:1	107.6	23.3
	20:1	65.5	26.1		20:1	90.3	24.2
3.6	3:1	115.5	12.7	15	3:1	124.7	19.1
	10:1	95.8	17.8		10:1	111.4	18.2
	20:1	92.8	29.2		20:1	93.9	30.8
7.5	3:1	129.1	18.1	25	3:1	116.4	17.2
	10:1	97.7	20.8		10:1	102.4	20.9
	20:1	91.2	24		20:1	96.6	26

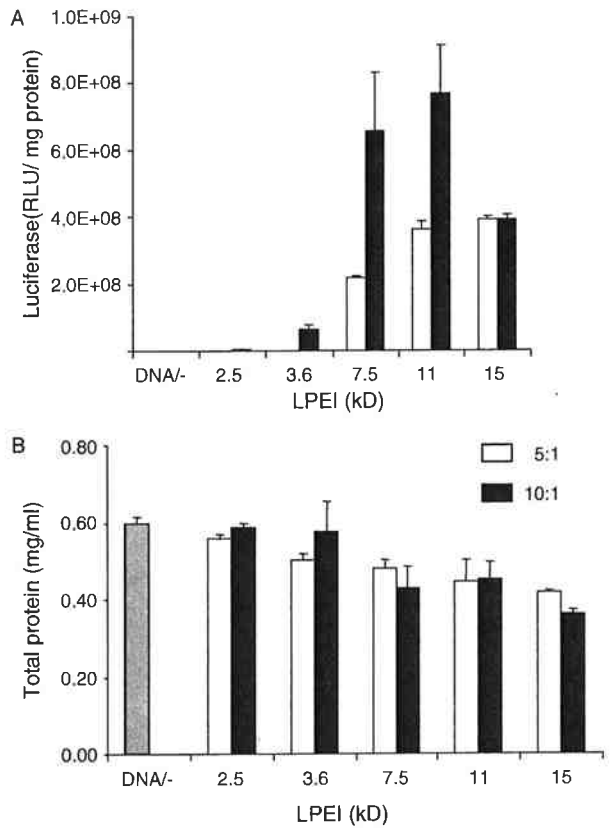

Fig. 2. Transfection activity (A) and cytotoxicity (B) of linear polyethylenimines. COS-1 cells were transfected with 1 μg of luciferase plasmid complexed with different linear polyethylenimines at 5 : 1 and 10 : 1 N : P ratio. Luciferase expression was quantified in cell lysate and expressed as relative light units/mg total protein. The cytotoxicity was measured by assaying for total cell protein.

to 7.5 kD, but this was accompanied with some cytotoxicity. LPEIs of molecular weight higher than 7.5 kD did not significantly add to transfection efficiency but led to increasing cytotoxicity. The N : P ratio of the LPEI-DNA complexes significantly influenced the transfection efficiency, with the higher N : P ratios resulting in higher transfection efficiency (Fig. 2A). These studies demonstrate a direct correlation between the molecular weight and biological activity of linear polyethylenimines. Based on these data, the different sized LPEIs may be divided into three groups. A low activity & low toxicity group comprising <3.6 kD LPEIs, a high activity & moderate toxicity group comprising 3.6 kD to 11 kD LPEIs, and a high activity & high toxicity group comprising >11 kD LPEIs. Accordingly, the 25 kD PEI falls in the high transfection and high toxicity group but equally effective (with lower cytotoxicity) lower molecular weight polyethylenimines (<10 kD) can serve as reasonable alternatives. Thus from these data we hypothesized that to achieve high efficiency and low toxicity the size of polyethylenimine backbone should be between 5–10 kD, and that it should be easily degradable into ≤3.6 kD fragments following transfection. To test this hypothesis we cross-linked a low molecular weight LPEI (3.6 kD) via disulfide linkage with the intent to obtain a medium size multi-block co-polymer (~5–10 kD) that would be efficient in gene delivery and easily degradable into smaller non-toxic fragments. The physico-chemical properties, transfection activity and cytotoxicity of this novel polymer are described below.

3.3. Cross-Linked Multi-Block Copolymer BD3.6K

The linear PEI 3.6 kD was chosen as the building block for the synthesis of cross-linked polymer BD3.6K due to its nominal cytotoxicity shown in *in vitro* studies. The monomer blocks were cross-linked by a dithiodipropionate ester linkage to promote degradation under physiological conditions. To minimize intra-molecular cross-linking and favor oligomer formation, most of the nitrogen atoms in the polymer backbone were blocked by BOC reagent. The BOC protected material was water soluble and amenable to chemical modification on the remaining free NH groups. The molecular weight of the cross-linked polymer, calculated from the mass to viscosity relationship (Fig. 1), was ~8.0 kD. The intrinsic viscosity actually measures effective gyrational radius of the polymer molecule, which is dependent on molecular mass and the shape of the molecule.[19] From the linear calibration curve it appears that LPEI molecules are tending to a "rod shape" in an aqueous solution of pH 2.5. For branch PEIs one has to incorporate a "shape factor" adjustment (>1), accounting for a more densely packed polymer molecule into the same gyrational radius. Hence, the actual molecular weight of the cross-linked polymer might be higher than the estimated value.

To further test if the polymer was cross-linked via disulfide bond, the cross-linked material was treated with reducing agent, $NaBH_4$, and the solution viscosity was measured. The molecular weight of the reduced polymer was calculated from the linear standard curve (Fig. 1). The monomer LPEI 3.6 kD was used as control in the reduction reaction. The $NaBH_4$ treatment resulted in the reduction of the solution viscosity from 2.57 to 2.2, which corresponds to polymer mass of 5 kD, a slightly higher value than that of its monomer block (3.6 kD), suggesting an incomplete degradation of the disulfide linkage by $NaBH_4$, which could be due to structural conformations that do not readily expose disulfide linkages.

3.4. Physico-Chemical Properties and Transfection Activity of Cross-Linked Multi-Block Copolymer

Addition of increasing amounts of BD3.6K to a fixed DNA concentration restricts DNA electrophoretic mobility within an agarose gel and demonstrates the formation of polymer-DNA complexes. At 1 : 1 N : P ratio the plasmid was not fully condensed and had a diameter of 256 nm. Increasing the N:P ratio to 5 : 1, 10 : 1, and 20 : 1 reduced the particle diameter to 130, 105, and 101 nm,

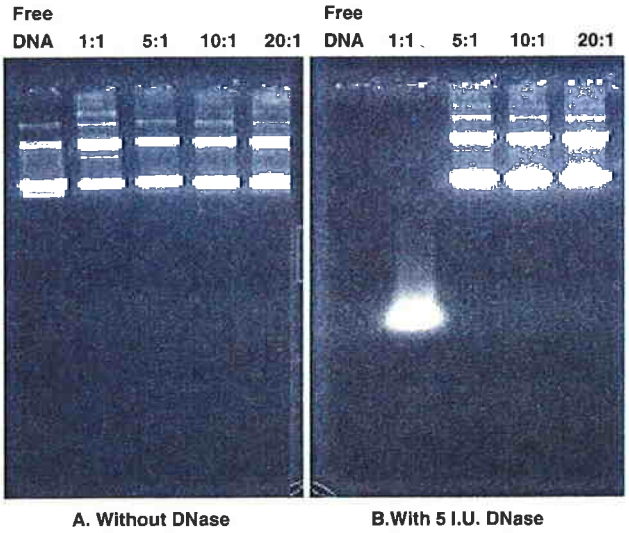

Fig. 3. Protection of LPEI complexed DNA against DNase action. The LPEI-DNA complexes prepared at various N : P ratios were incubated with 5 IU of DNase I in digestion buffer (40 mM Tris-HCl, 10 mM NaCl, 5 mM $MgSO_4$) for 60 minutes at 37 °C. The reaction was terminated with an equal volume of termination buffer and DNA was dissociated from the polymer by dextran sulfate treatment. The DNA integrity was examined following electrophoretic separation of the mixture at 100 V for one hour on agarose gel.

respectively (Fig. 4A). The zeta potential values at 1 : 1, 5 : 1, 10 : 1, 20 : 1 N : P ratios were −48.4 mV, 24.2 mV, 26.6 mV, and 28.7 mV, respectively (Fig. 4B). To determine if DNA complexation with BD3.6K protected the DNA from degradation, the complexes were incubated with 5 IU DNase for 60 min, decomplexed by addition of dextran sulfate and separated by gel electrophoresis. As shown in Figure 3B, the unformulated DNA or partially complexed DNA (N : P 1 : 1) was totally degraded while fully complexed DNA remained stable during the DNase incubation.

The ability of BD3.6K to promote gene transfer in cell culture was examined with a luciferase plasmid. As shown in Figure 5A, the luciferase expression with the cross-linked polymer was about 20 fold higher than that of its monomer. The transfection efficiency of the cross-linked polymer determined by β-galactosidase assay was 75–90% (Fig. 5B). The transfection activity and cytotoxicity of BD3.6K was compared to that of the LPEI series (2.5–25 kD). The transfection activity of BD3.6K was superior to all higher molecular weight test polymers (7.5–25 kD), while the cytotoxicity was considerably lower (Fig. 6). The transfection activity and cytotoxicity of BD3.6K was also compared with ExGen 500, a commercially available ~25 kD polyethylenimine. The luciferase expression from BD3.6K and ExGen 500 transfected COS-1 cells was 2.06× 10^9 RLU/mg protein and 1.03× 10^9 RLU/mg protein, respectively. The total protein level was 0.27 mg/ml and 0.18 mg/ml, respectively.

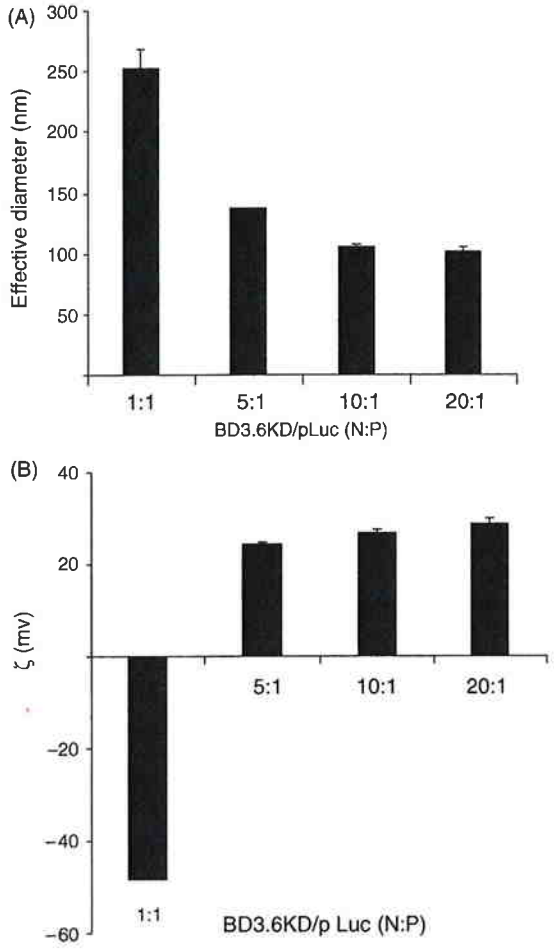

Fig. 4. Particle size (A) and zeta potential (B) of BD3.6K-DNA complexes. pCMV-luciferase was formulated with multi-block copolymer, BD3.6K, at different N : P ratios in 10% lactose solution. The particle size and zeta potential of the nanocomplexes in Milli-Q water was determined at 657 nm at a constant angle of 90° with 90Plus/BI-MAS Particle sizer.

To determine if BD3.6K can mediate gene transfer *in vivo* the BD3.6K-luciferase plasmid complexes prepared at two different N : P ratios were administered into mice by tail vein injection and luciferase expression was quantified in the lungs. While there was significant lung gene transfer in BD3.6K-luciferase treated animals there was no discernable expression from 3.6 kD monomer (Fig. 7). We next evaluated BD3.6K transfection efficiency and toxicity in comparison with 25 kD PEIs (branched and linear) following intravenous injection. Luciferase expression in lungs and serum chemistry analysis was conducted 24 hrs after iv treatment. As can be seen in Figure 8, BD3.6K at 60 : 1 N : P, showed a significant increase (2.5 folds) in luciferase expression level in lungs relative to linear 25 kD PEIs (20 : 1), (N : P). In addition, a 70-fold increase in luciferase levels compared to branched 25 kD PEI at (20 : 1), (N : P). Gross observation indicated no overt toxicity on any group except the branched group where two of five animals died following intravenous administration. Serum

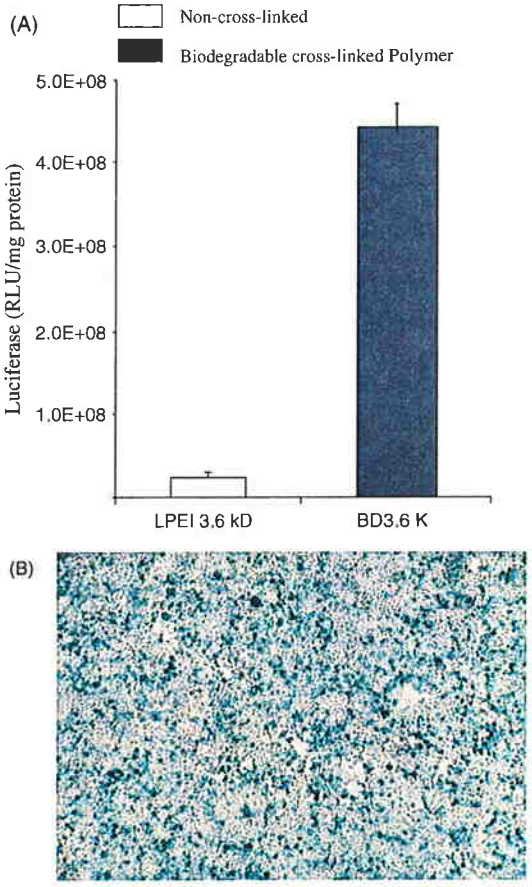

Fig. 5. *In vitro* gene transfer by BD3.6K. (A) HEK 293 cells were transfected with 1 μg of luciferase plasmid complexed with BD3.6K or monomer LPEI 3.6 kD at 20 : 1 N : P ratio. Luciferase expression was quantified in cell lysate and expressed as relative light units/mg total protein. (B) X-gal stain of HEK 293 cells transfected with BD3.6K-β-galactosidase complexes.

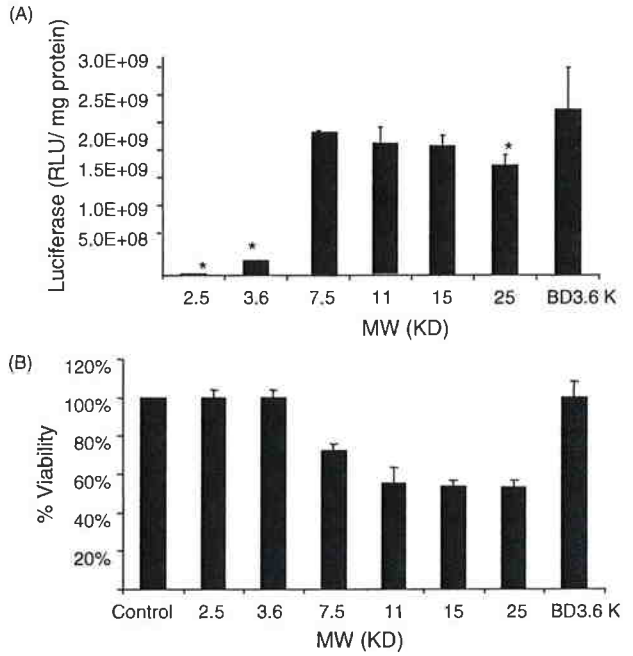

Fig. 6. *In vitro* transfection activity (A) and cytotoxicity (B) of BD3.6K. COS-1 cells were transfected with 1 μg (12-well plate) or 0.1 μg (96-well plate) of luciferase plasmid complexed with different size linear polyethylenimines and BD3.6K at 20 : 1 N : P ratio. Luciferase expression was quantified in cell lysate and expressed as relative light units/mg total protein. The cytotoxicity of polymer-DNA complexes was determined by addition of 20 μL of CellTiter 96® reagent to each well after transfection and measuring optical density of the colored product at 490 nm. The cell viability was calculated according to manufacturer's instructions. (*) indicates significant difference from BD3.6K. $p < 0.02$ for 25 kD and $p < 0.001$ for 2.5 kD and 3.6 kD.

chemistry analysis was performed; results are summarized in Figure 9. Glucose levels in all treated animals were significantly lower compared to untreated group ($p < 0.05$) and that could be due to the result of loss of appetites following intravenous administration. Additionally, levels of AST and ALT were increased in all treated animals especially in branched 25 kD PEI ($p < 0.05$ in comparison with BD3.6K) indicating of some liver toxicity particularly with branched PEI. Other measured parameters were generally unchanged. Importantly, very similar chemistry profiles were noted for BD3.6K (60 : 1) and LPEI 25 kD (20 : 1), (N : P), however significant expression advantage was associated with BD3.6K. These data demonstrate the significant advantage of using a cross-linked BD3.6K over the commercially available high molecular weight PEI polymers for *in vivo* gene delivery. The improvement in systemic tolerability of polyethylenimines was achieved by accelerating its molecular degradation, which suggests that slow clearance from target cells might be an important contributing factor to the systemic toxicity of PEI. A small but significant induction of liver toxicity in BD3.6K treated groups at higher polymer load (N/P 60) (Fig. 9) warrants the need for incorporation of additional strategies in the polymer design. Strategies including the attachment of targeting ligands and reduction in the particle charge

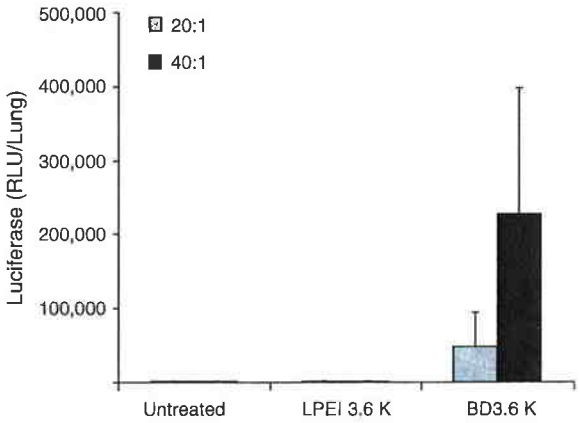

Fig. 7. *In vivo* transfection activity of BD3.6K. The transfection complexes containing luciferase plasmid and BD3.6K were administered into the tail vein of ICR mice. The plasmid concentration in the formulation was fixed at 0.1 mg/ml to yield a 30 μg dose in 0.3 ml injection volume. Lungs were harvested 24 hours after gene injection, homogenized in 1 ml TENT buffer and assayed for luciferase activity. Values are means ± SD, $n = 5$.

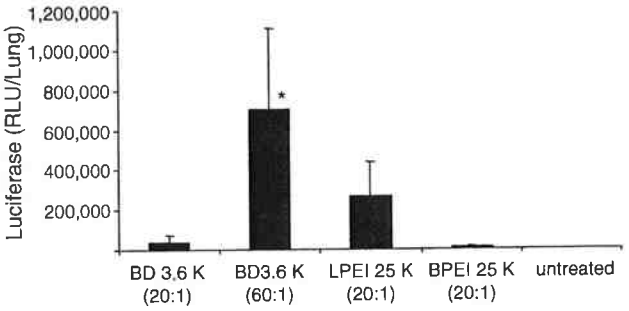

Fig. 8. Comparison of the *in vivo* transfection activity of BD3.6K, linear PEI (LPEI), and branched PEI (BPEI). 30 μg of plasmid DNA encoding luciferase enzyme in 0.3 ml injection volume was administered into the tail vein of ICR mice. Lungs were harvested 24 hours after administration Values are means $\pm$ SD ($n = 5$) in all groups except for BPEI 25 kD, $n = 3$. (*) indicates significantly different from all other treatment groups.

density to improve delivery specificity and *in vivo* stability are being investigated in our laboratory.

The degradable cross-linked copolymer BD3.6K is distinct from the branch PEI-based multi-block copolymers[20] with respect to water solubility and transfection activity. BD3.6K is highly soluble in aqueous solution and transfectionally superior to its monomer whereas the branch PEI-based multi-block polymers are poorly soluble in aqueous solution and only marginally better than their corresponding monomers in transfection activity.[20] The difference in transfection activity between BD3.6K and that of the branch PEI-based polymers is presumably due to polymer design, synthesis scheme and/or physiochemical properties. For example, the linker to PEI molar ratio in the branch PEI based copolymers was ≥ 1[20] which shows a significant dilution of the polymer backbone and could be the reason for marginal improvement in transfection activity. In comparison, the linker to PEI ratio in BD3.6K was <0.2. Additionally, the branch PEI would favor intra-molecular looping as opposed to inter-molecular cross-linking and that could be a potential reason for its poor solubility. We believe the introduction of BOC protection in the synthesis scheme is the underlying reason for high aqueous solubility of BD3.6K, as it would minimize polymer branching by preventing intra-molecular looping. A hydrophobically modified cross-linked branch PEI of low molecular weight has been recently reported.[21] The synthesis of this cross-linked branch PEI uses disulfide linking reagents specific to the primary amines of the branch PEI and requires non-preparative laborious purification of the product by gel-permeation chromatography.[21] The procedures used in the preparation of BD 3.6K do not pose these limitations.

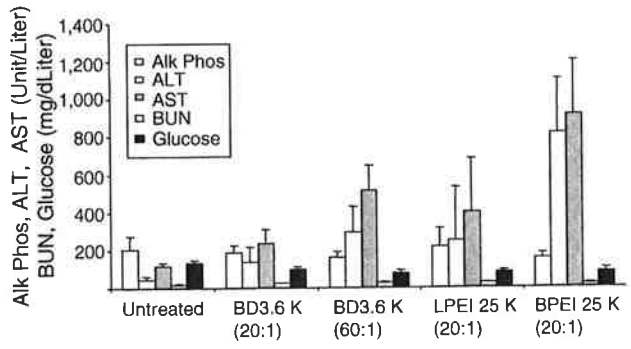

Fig. 9. Serum chemistry analysis 24 hrs following tail injection of ICR mice. Serum was collected from same animals used for luciferase expression in Figure 8. Alkaline Phosphatase (ALK), Alanine Aminotransferase (ALT), Aspartame Aminotransferase (AST), Blood Urea Nitrogen (BUN). All values are expressed as mean $\pm$ SD ($n = 5$) except for BPEI 25 kD where $n = 3$.

4. CONCLUSIONS

A series of low molecular weight linear polyethylenimines (2.5–25 kD) were synthesized and evaluated for transfection efficiency and cytotoxicity. A single uniform procedure was used for the synthesis to minimize structural variability normally associated with the commercial preparations of branched polyethylenimines. The size of the polymer backbone has a profound effect on the transfection activity and cytotoxicity of the linear polyethylenimines. High molecular weight LPEIs exhibited higher transfection activity and higher cytotoxicity, while lower molecular weight LPEIs exhibited lower transfection activity and lower cytotoxicity. The transfection activity of the test series reached its maximal value at around 7.5 kD, further increases in the polymer molecular weight (up to 25 kD) did not improve transfection activity but exerted higher cytotoxicity. These results demonstrate that the acute toxicity observed with the use of 25 kD PEI can be reduced by using the lower molecular weight polyethylenimines (5–10 kD) or extending the size of the low molecular weight polyethylenimines to 5–10 kD by controlled intermolecular cross-linking. Cross-linkage of a non-toxic low molecular weight LPEI (3.6 kD) into an oligomer (8 kD) via degradable linkage significantly improved the polymer transfection efficiency or tolerability as compared to the monomer block or 25 kD linear or branched PEI, demonstrating the feasibility of this approach. These low molecular weight cross-linked copolymers and their functionalized derivatives may serve as useful tools for delivery of therapeutic nucleic acids including plasmid, oligonucleotides, and inhibitory RNA.

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